

Development of Animal-Based Bacterial Biosensors for the Detection of Estrogenic Compounds

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Abstract

Estrogenic compounds are an important class of chemicals that have the ability to modulate the function of the nuclear hormone receptor, estrogen receptor beta (ER β). These compounds can be found naturally in the environment or chemically synthesized, and have been linked to health risks ranging from altered sexual development to breast cancer. Previous work in this lab resulted in an *Escherichia coli* cellular biosensor comprised of an engineered protein scaffold with an inserted human ER β ligand-binding domain fused to a thymidylate synthase reporter enzyme. Through growth in thymineless media, the human ER β biosensor can distinguish between estrogen agonists and antagonists based on the resulting *E. coli* growth phenotype. Preliminary results using several animal-based ER β bacterial biosensors (cow, zebrafish, and rat) revealed species-specific responses to ligands and identified chlorodecone as an ER antagonist and dioctyl phthalate and Bisphenol B as weak ER agonists. This research sought to extend the ER β biosensor method to include sentinel animal species, such as the zebra finch and wood frog, which serve as early indicators of the presence of environmental toxins. Using bioinformatics and molecular cloning techniques, novel sentinel species-based ER β biosensors were created and validated against known strong and weak estrogens using the high throughput bacterial assay method. Higher sensitivity analogs of four animal biosensors were also constructed and exhibited greater than 10-fold increases in ligand response sensitivity. Unique responses to weak and strong estrogens were also observed for biosensors across a range of vertebrates (mammals, fish, birds, and amphibians), suggesting species-specific susceptibility for certain endocrine disruptors with greater deviations observed for weaker estrogens. In addition, biosensors selective for TR β and

ER α were constructed, exhibiting the ability of the ER β biosensor to be easily modified for other pharmaceutically relevant nuclear hormone receptors. Adaptation of the ER β biosensor to sentinel species, in particular, will enable the prevention and treatment of diseases through early detection of estrogenic compounds in the environment and expand the bacterial biosensor library. These animal ER β bacterial biosensors will also provide a broader understanding of how drug response deviates across species in pre-clinical development.

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Introduction

Estrogens are hormones that are involved in critical metabolic pathways found in the reproductive, nervous, cardiovascular, bone, and hematopoiesis systems [1].

Estrogens can be found naturally in the environment or artificially synthesized, and through activation of one of its nuclear hormone receptor subtypes (estrogen receptor beta or estrogen receptor alpha), estrogen plays a key role in human development and the onset of puberty. However, estrogen-like endocrine disrupting compounds (EEDCs), which are primarily synthetic estrogenic compounds, can interfere with the activity of natural estrogens due to their structural similarity as seen in Figure 1. Commonly well-known EEDCs include chemicals such as DDT, dioxins, PCBs (polychlorinated biphenyls), BPA, PBBs (polybrominated biphenyls), and phthalate esters [2].

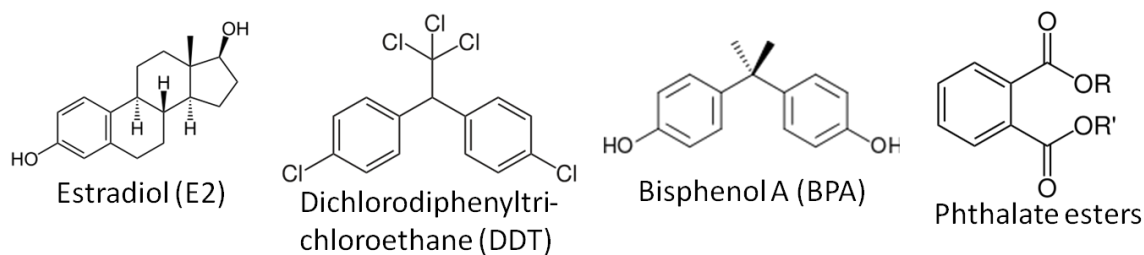


Figure 1: Chemical structure for E2, the native ligand for the estrogen receptor, along with the structures of several well-known EEDCS

One of the most well-known cases of EEDC interference is DES and its impact on the female fetuses of mothers who took the drug from 1948 to 1971. These children later suffered from reproductive organ dysfunction, immune system disorders, abnormal pregnancies, and experienced increased rates of vaginal clear-cell adenocarcinomas as young women [3]. In addition to human impacts, estrogenic compounds can also have detrimental effects on wildlife as evidenced by studies documenting distorted sex organ

development and function in alligators due to pesticide exposure as eggs, DDT-induced thinning of eagle egg shells, demasculinization and feminization of fish, birds, and mammals, and impaired immune systems in birds and mammals [3,4].

Although estrogenic compounds can result in human and animal health risks, some can also act as potential pharmaceuticals due to their ability to modulate the activity of the estrogen receptor as selective estrogen receptor modulators (SERMs). For example, tamoxifen, an estrogen antagonist, is the most commonly prescribed endocrine treatment for hormone receptor-positive breast cancer [5]. Furthermore, raloxifene (another SERM) and additional hormone therapies have also been approved for the prevention and treatment of postmenopausal osteoporosis, with other SERMs in development or in clinical trials [6]. Thus, estrogenic compounds and other endocrine disruptors are promising pharmaceutical candidates for nuclear hormone receptor (NHR) related diseases.

However, with the comprehensive cost of drug development and discovery estimated at US \$1.7 billion per drug, major pharmaceutical companies such as Sanofi and Astra-Zeneca are spending exorbitant amounts annually on new drugs [7]. This cost is attributed primarily to the high rate of failure of trial compounds throughout the drug development process caused by a lack of efficacy. Only 11% of compounds that initiate first-in-man studies are successfully registered and approximately 43% of compounds in phase III trials and 23% in registration fail [7]. As a result, drug development is time-consuming, expensive, and difficult for companies, greatly reducing the number of novel therapeutics introduced per year.

One of the contributors to the high cost and time requirement of drug development is the immense number of animal models required during preliminary studies. Current models for identifying ligands with the potential to affect NHR function involve whole animal or mammalian cell assays as well-established protocols exist for rats and mice. A commonly used procedure for assessing estrogenic compounds is the rat uterotrophic assay in which 21-22 days old rats are fed three daily doses of the compound of interest. The rats are then killed on the fourth day and the absolute uterus weight is determined [8]. Procedures for other vertebrates follow a similar model of repeatedly exposing the animal to a drug for a specific duration of time and then killing them and measuring the growth of sex organs [9]. Many of these models also involve large sample sizes of animals, require specialized training for animal handling and care, and take weeks in order for the animals to reach the ideal testing age. Thus, while these models are the most reliable in the prediction of endocrine disrupting compounds due to the complexity of using a living animal, they are also time-consuming, labor-intensive, and impractical for high-throughput screening [10].

In addition to animal models, pharmaceutical companies also utilize several other methods, such as high-throughput screening, focused screening, high-throughput docking and pharmacophore-based screening algorithms, and physiological screening to find potential hits [11, 12]. High-throughput screening typically involves screening millions of compounds in 384- to 1536-well microtiter plates over several weeks using mammalian cell lines that express the desired receptor or drug target [11]. Once a library of hits has been compiled, these compounds are then tested in additional assays to create dose response curves and eliminate hits that exhibit non-reversible behavior.

Another technique gaining popularity is high-throughput docking and pharmacophore-based screening algorithms (virtual screening). Virtual screening consists of using computational models to predict binding affinity and enables the ability to test compounds that a researcher does not physically possess. Pharmacophore searching algorithms and cheminformatics are also performed as complements to high-throughput docking, improving computational efficiency through the ability to generate large virtual libraries of compounds, perform extensive filtering of the libraries, and automate data processing [12]. Thus, through *in silico* screening techniques, it is possible to easily identify small subsets of compounds for testing as compared to screening millions of chemicals. Currently though, these techniques are primarily used as an accompaniment to bioassays as many commercially available high-throughput docking programs operate using a rigid receptor approximation and thus cannot account for the flexibility of the protein or ligand in determining a ligand's binding ability [12].

Finding a way to better facilitate pharmaceutical innovation through the creation of biomarkers capable of accurately determining compound efficacy in the preliminary developmental stages would reduce clinical costs and could lead to an increase in the amount of therapeutics introduced for the treatment of diseases. Furthermore, an assay that could determine potential endocrine disruptors using bacterial biosensors would provide an alternative method to preliminary animal models, decreasing the amount of time, money, and animal lives that must be sacrificed during the drug development process. A high-throughput assay utilizing bacterial biosensing cells would also be faster and cheaper than mammalian cells if it could be shown that the response was comparable across both cell systems. In addition, using bacterial biosensors would enable the

determination of hits and their dose response curves with only a single assay. *Escherichia coli* is also a well-characterized and simple organism to modify, allowing for facile adaptation of the assay for a wide range of drug targets. Finally, development of animal-based bacterial biosensors will provide a greater understanding of how drug response may deviate across species during preclinical trials and can help reduce human and wildlife exposure to endocrine disruptors through early detection of these compounds in the environment.

Background

Nuclear Hormone Receptors

Nuclear hormone receptors (NHRs) are proteins that act as ligand induced transcription factors during gene expression and exist in a wide variety of metazoan organisms [13]. NHRs are considered a single superfamily of receptors, but consist of multiple subfamilies that are defined based on sequence homology and on dimerization and DNA-binding mechanism [14]. For example, subfamily 3 consists of the estrogen receptor-like receptors and includes amongst its members, estrogen receptors α and β (ER α and ER β), the glucocorticoid receptor, and the androgen receptor. The NHRs consist of different regions with independent functional domains that can be interchanged amongst related receptors without any loss of function. All of the receptors are comprised of a variable N terminus, a conserved DNA-binding domain (DBD), a linker region, a conserved ligand-binding domain (LBD), and a variable C terminus as shown in Figure 2 on the next page.

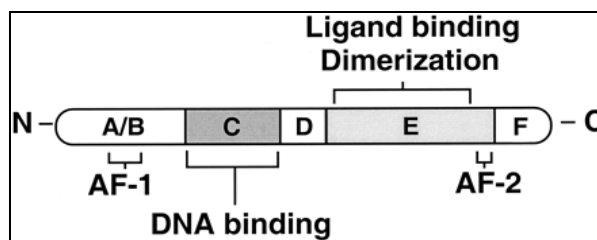


Figure 2: General schematic of the domains in a nuclear hormone receptor [13]

Due to the interaction between the DBD and LBD, small molecules can be used to control the transcription activity of the NHR. Compounds that have the ability to bind to the NHR and activate transcription are termed agonists, while compounds that can inhibit agonist-induced responses are called antagonists [10]. As receptors have both an inactive and an active state, agonists are able to activate a receptor by driving equilibrium to favor the receptor's active state. Conversely, antagonists block agonist responses by binding to the receptor and driving equilibrium to favor the inactive state [15]. Due to their ability to regulate a variety of physiological and developmental functions, ranging from reproduction to metabolism, nuclear hormone receptor function has been linked to various human diseases such as cancer, obesity, and diabetes [16]. Thus, NHRs are an important class of protein targets for drug discovery as identifying compounds with the ability to modulate the function of these nuclear hormone receptors offers an opportunity for the development of new and effective therapeutics.

Bacterial Biosensor Construction

Bacterial biosensors are microorganisms that have been engineered to express a biosensing protein that binds to a target compound/ligand. The presence of the target compound causes a change in the cell's activity, which can be read through a change in growth rate or fluorescence [17]. The *E. coli* hormone biosensor used as a template for the sentinel species-based biosensors consists of an engineered protein scaffold with four

unique protein domains: a maltose-binding domain, a cleaving and splicing-deficient domain from the *Mycobacterium tuberculosis* RecA intein (mini-intein), the ligand-binding domain (LBD) of estrogen receptor beta (ER β), and a thymidylate synthase (TS) reporter enzyme. A schematic of the biosensor can be found in Figure 3 on the next page.

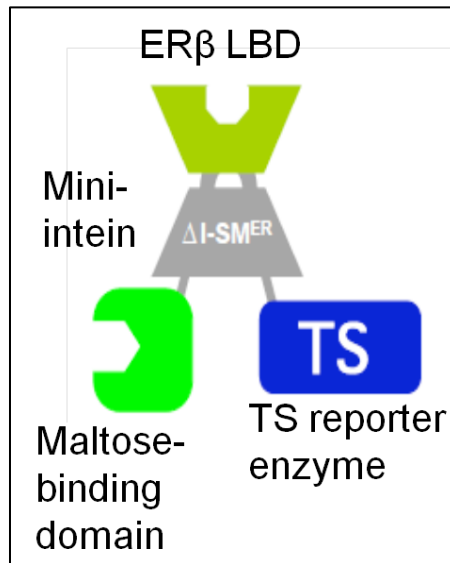


Figure 3: Diagram of the biosensor

The maltose-binding domain serves to enhance solubility of the fusion protein while the mini-intein assists in stabilizing the NHR LBD, as correct folding of nuclear hormone receptors has been found to be difficult in *E. coli* [10]. Finally inclusion of the NHR LBD and TS into the biosensor scaffold enables the ability to create a high-throughput assay for ligand identification by directly coupling *E. coli* growth to ligand binding.

Biosensor mechanism

All of the biosensors were constructed using *E. coli* D1210 Δ thyA, a bacterial strain that has the native thymidylate synthase (TS) enzyme function knocked-out [18]. Therefore, the bacteria are unable to synthesize thymidine monophosphate (dTMP), and

thus cannot create new DNA and replicate in thymineless media. Usage of the knockout strain ensures that any observed bacterial growth in the presence of thymineless media must be the result of endogenously synthesized thymine from the activation of the TS reporter enzyme located in the biosensor scaffold.

The thymidylate synthase reporter enzyme is involved in the folate cycle and is necessary for DNA synthesis and survival of the cell in the absence of thymine. Thymidylate synthase exists as a homodimer and catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) by methylene tetrahydrofolate to produce dihydrofolate and dTMP, the precursor to thymine as seen in Figure 4 below [19].

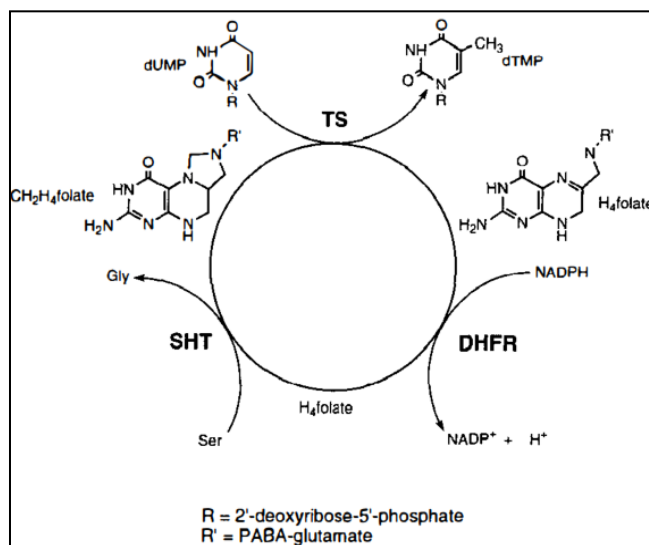


Figure 4: The dTMP cycle [19]

In the bacterial biosensor, the TS reporter enzyme is fused to both the maltose-binding domain and the mini-intein, which contains the LBD as seen in Figure 2. Thus, it is hypothesized that in the protein's natural state, with no ligand bound, the dimerization of TS is partially blocked causing low TS activity [10]. When a ligand is bound to the ligand binding pocket of the LBD though, it induces a conformational change within the protein [13]. Through this conformational change, the LBD can inhibit TS activity by

blocking TS dimerization such that even lower activity than the unbound state is observed [10]. A ligand can also induce a conformational change in the LBD that allows for increased TS activity. Therefore, agonist and antagonist ligands can be determined through TS activity level and the resulting growth rate of the cell.

Methods

Chemicals and Reagents

The estrogen analogs 17 β -estradiol (E2), Estriol (E3), Estrone (E1), Diethylstilbestrol (DES), Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), Bisphenol A (4,4'-(propane-2,2-diyl)diphenol; BPA), Bisphenol S (4,4'-sulfonyldiphenol; BPS), and Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) were purchased from Sigma-Aldrich (St. Louis, MO). In addition, 15 ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) recommended compounds were also purchased from Sigma-Aldrich (St. Louis, MO) and a list of these compounds can be found in Table 1 below. All compounds were dissolved in dimethyl sulfoxide (DMSO) to a final stock concentration of 10 mM and stored at -20 °C.

Table 1: List of the 15 tested ICCVAM recommended compounds

1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene	Linuron
Diethyl phthalate	2,4,5-Trichlorophenoxyacetic acid
Chlorodecone	3,4-Cumyl phenol
Spironolactone	6-Propyl-2-thiouracil
4,4'-(butane-2,2-diyl)diphenol (Bisphenol B)	Atrazine
Cycloheximide	Cyproterone acetate
Progesterone	Flavone

The thymineless (-Thy) media used in all of the biosensor tests consisted of 1 mL of 0.1 M CaCl₂, 200 mL of Minimal Davis Broth (35 mg/mL dipotassium phosphate, 10

mg/mL monopotassium phosphate, 2.5 mg/mL sodium citrate, 0.5 mg/mL magnesium sulfate, 5 mg/ mL ammonium sulfate), 10 mL of 10% casamino solution, 10 mL of 20% glucose, 200 μ L of 1% Thiamine HCl, 10 mL of Thy Pool solution (2 mg/mL of each of the following: L-Arg, L-His, L-Leu, L-Meth, L-Pro, and L-Thr), 4 mL of 25 mg/mL ampicillin and q.s. to 1 L using deionized water. The Minimal Davis Broth and 0.1 M CaCl_2 were sterilized by autoclaving at 121 $^{\circ}\text{C}$ for 40 minutes and then cooled to room temperature. The other stock solutions were sterilized via vacuum filtration using a 0.2 μm filter.

Plasmid Construction

Due to the modular nature of the biosensor and the conservation of the estrogen receptor across species, sentinel-species based $\text{ER}\beta$ sensors could easily be constructed through replacement of the human $\text{ER}\beta$ ligand-binding domain (LBD) with the $\text{ER}\beta$ LBD of each respective species. The putative LBD of each species of interest was determined through protein sequence alignments of each animal's $\text{ER}\beta$ against human $\text{ER}\beta$ as seen in Figure 5 below.

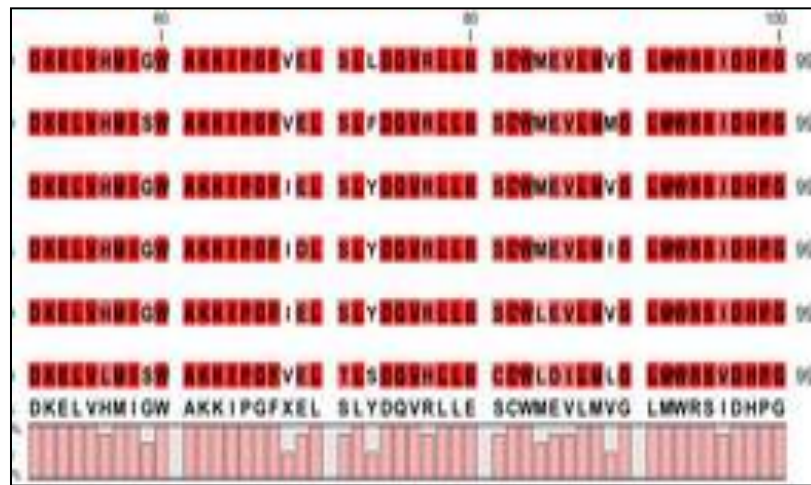


Figure 5: Protein sequence alignment of $\text{ER}\beta$ LBDs with the alignment order as follows from the top: rat, human, alligator, zebra finch, green anole lizard, and zebrafish.

Once the ER β LBD sequences of the zebrafish and the American alligator had been determined, the corresponding DNA sequences were synthesized from IDT technologies (Coralville, IA). In addition, cDNAs of the estrogen receptors of the wood frog (*L. sylvaticus*) and the northern leopard frog (*L. pipens*) were also obtained. The ER β LBDs of the three sentinel species (zebrafish, alligator, and wood frog), were then cloned using overlap extension PCR into both the standard pMIT vector and the pMIT:1GS (more sensitive) vector previously designed and built in the lab. This technique was chosen as a more efficient method to avoid the need to subclone the LBDs into a shuttle vector such as pGEM and to make the method more generalizable regardless of the species. Two pairs of PCR primers (FAgeI/R110; F383/RXhoI) were designed and used to amplify the N- and C-terminus intein fragments. The 110_383 cloning sites were chosen to maintain consistency with the original cloning of pMIT::ER β *(human). After the two intein fragments had been successfully amplified via PCR, primers for amplifying the LBDs were designed as this cloning method requires the primers to be long enough such that they amplify 20 base pairs of the intein in addition to the desired LBD. From there, a final round of PCR was completed to incorporate all three PCR fragments into a single unit using the outer-most primers of FAgeI and RXhoI. Q5 polymerase was also used for all PCR reactions due to its high-fidelity. The resulting PCR products, as well as the pMIT vectors, were digested with SgrA1 (a unique restriction enzyme site within the intein's N terminal) and XhoI. Ligations were prepared at room temperature and Anoue DH5 α competent cells were used for the transformations. Cloning was confirmed via digest check and PMGF sequencing.

High-Throughput 96 -well Plate Biosensor Assay

As described in previous work, pMIT::ER β *(animal) plasmids were transformed into the *E. coli* strain D1210 Δ thyA::Kan^R [F⁻ Δ (gpt-proA)62 *leuB6 supE44 ara-14 galK2 lacY1* Δ (mcrC-mrr) *rpsL20 (Str^r) xyl-5 mtl-1 recA13 lacI^q*] and selected for on Luria-Bertani (LB) agar plates supplemented with 200 μ g/mL ampicillin and 50 μ g/mL thymine. Single colonies were chosen and used to inoculate 3 mL LB media liquid cultures supplemented with 200 μ g/mL ampicillin and 50 μ g/mL thymine, and then incubated with shaking at 37°C for 14-16 hours. A portion of the overnight cultures (30 μ L) were then used to inoculate fresh 3 mL LB media liquid cultures supplemented with 200 μ g/mL ampicillin and 50 μ g/mL thymine. The liquid cultures were incubated and shaken at 37°C until an optical density at 600 nm (OD₆₀₀) of approximately 1.1 -1.3 was reached.

The LB cultures were then diluted into -Thy media and 198 μ L of the diluted biosensor cells were dispensed into each well of a 96-well plate. The dilution factor of the LB cultures into the -Thy media was specifically adjusted to each biosensing strain to account for the strain's basal growth. These adjusted dilutions, in terms of LB inoculum to -Thy media (v/v), were 1:400 for pMIT::ER β *(pig), 1:800 for all of the pMIT::ER β 1GS*(animal) biosensors, and 1:200 for the remaining pMIT::ER β * (animal) biosensors.

Each ligand of interest was diluted into DMSO in a 96-well plate from a stock concentration of 10 mM to a maximum concentration of 0.1 mM and a minimum concentration of 2.56E-10 M for a 5-fold serial dilution, and a maximum concentration of 0.1 mM and a minimum concentration of 1E-12 M for a 10-fold serial dilution. The last

column of each serial dilution was pure DMSO to provide a baseline value for biosensor growth without ligand added. The ligand was then introduced to the diluted biosensor cells by adding 2 μL of each dilution to each well for a total volume of 200 μL of ligand and cells per well. Both this step and the distribution of diluted biosensor cells into the 96-well plate were performed using either a multi-channel pipette or a robotic liquid handling station (BioMek, 2000/3000, Beckman Coulter). The plates were then incubated with shaking at 34°C for up to 22 hours. The OD_{600} of each well was measured using a UV-vis plate reader (BioTek Synergy2) every two hours starting at 14 hours after initial incubation. A schematic of the assay process can be seen in Figure 6 on the next page.

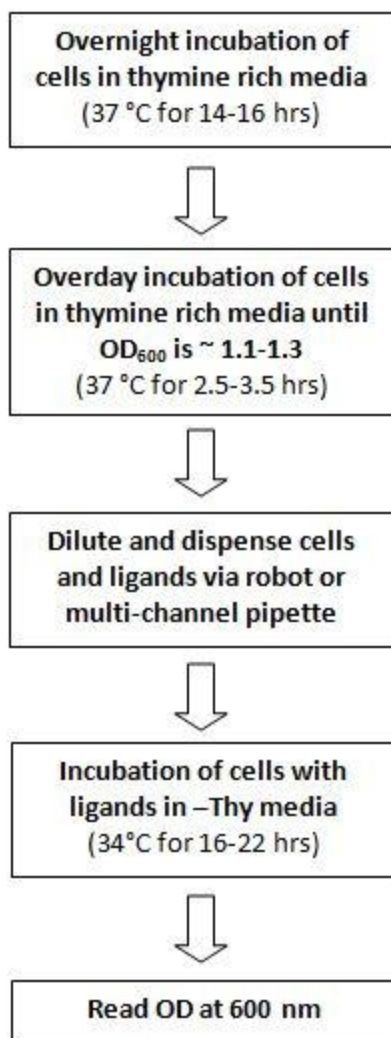


Figure 6: Flowsheet of the steps of the high throughput bacterial biosensor assay

Determination of EC₅₀ and RPTA values

The effectiveness and strength of each ligand was determined through calculation of its EC₅₀ value, which is the concentration at which 50% of the maximum response is achieved [16]. The raw OD₆₀₀ absorbance data was normalized to a percentage of the response of the maximum test concentration after first subtracting the background signal. EC₅₀ values were then calculated using an Excel-based program that fit the normalized absorbance responses to the Hill equation shown in Equation 1 on the next page where Y is defined as the scaled OD₆₀₀ and X is the log of the ligand concentration.

$$Y = b + \frac{a-b}{1+d*10^{c-x}} \quad (1)$$

The other four parameters (a, b, c, and d) were determined using non-linear regression where c is defined as the log of the EC₅₀ value for the ligand and a and b are the highest and lowest OD₆₀₀ values for a single compound respectively.

The relative estrogenicity of a compound as compared to E2 was also determined through calculation of relative pseudotransactivation values (RPTA) where RPTA is defined as $RPTA = \frac{EC_{50}^{E2}}{EC_{50}^{ligand}}$ and % RPTA is defined as RPTA x 100% [20].

Results

Determination of estrogen agonists and antagonists

In order to test the ability of the animal-based biosensors to detect estrogenic compounds, 15 ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods)-recommended compounds were screened against four different animal biosensors consisting of human, two additional mammals (cow and rat), and a fish (zebrafish). Each biosensor test consisted of 5 ICCVAM recommended compounds, a positive control of E2 (native ligand for ER β), a negative control of Triac (thyroid hormone), and a known weak ER agonist (BPA). Dose-response curves were then generated using the OD₆₀₀ data gathered 16-22 hours after ligand introduction and the responses of the four animal biosensors against seven compounds can be seen in Figures 7-10 on the following pages.

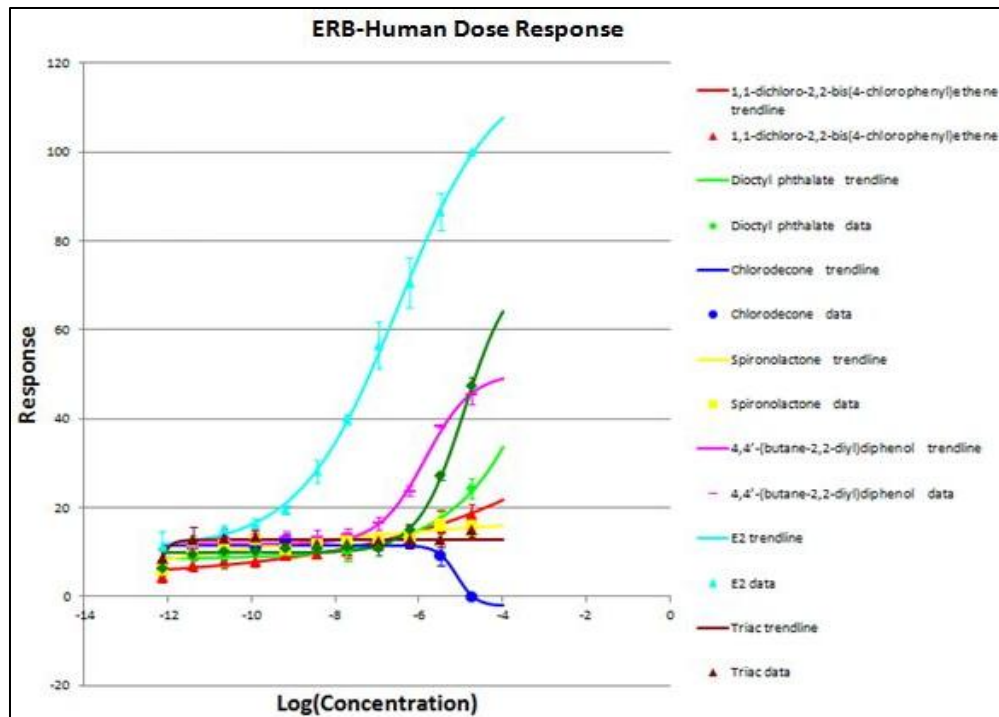


Figure 7: Dose response curve for ER β -Human

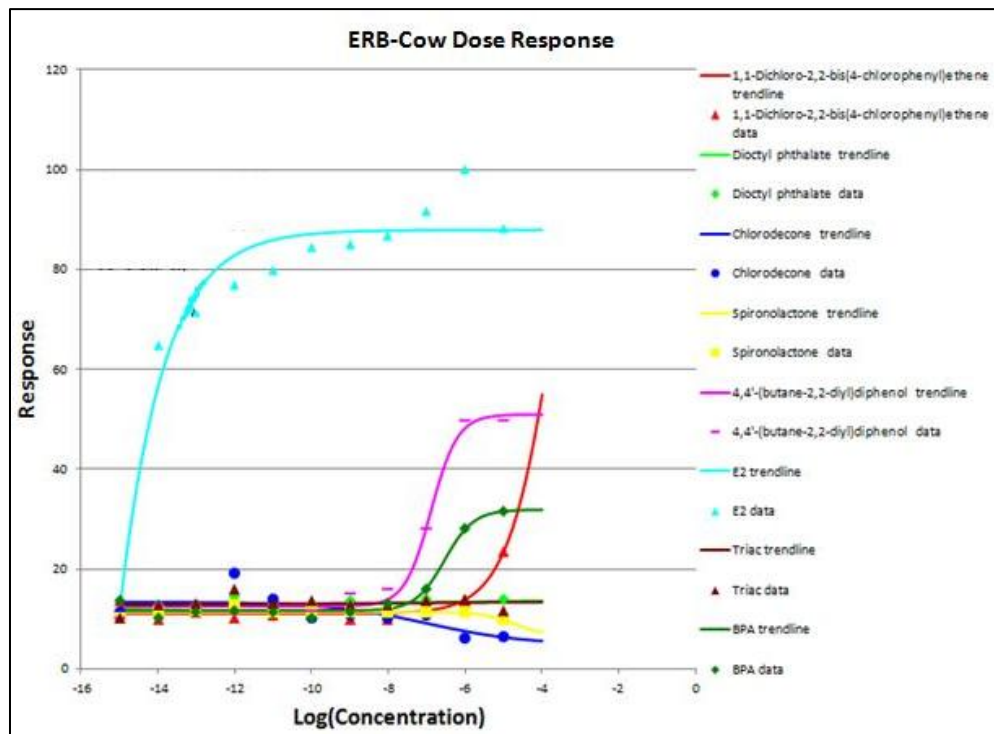


Figure 8: Dose response curve for ER β -Cow

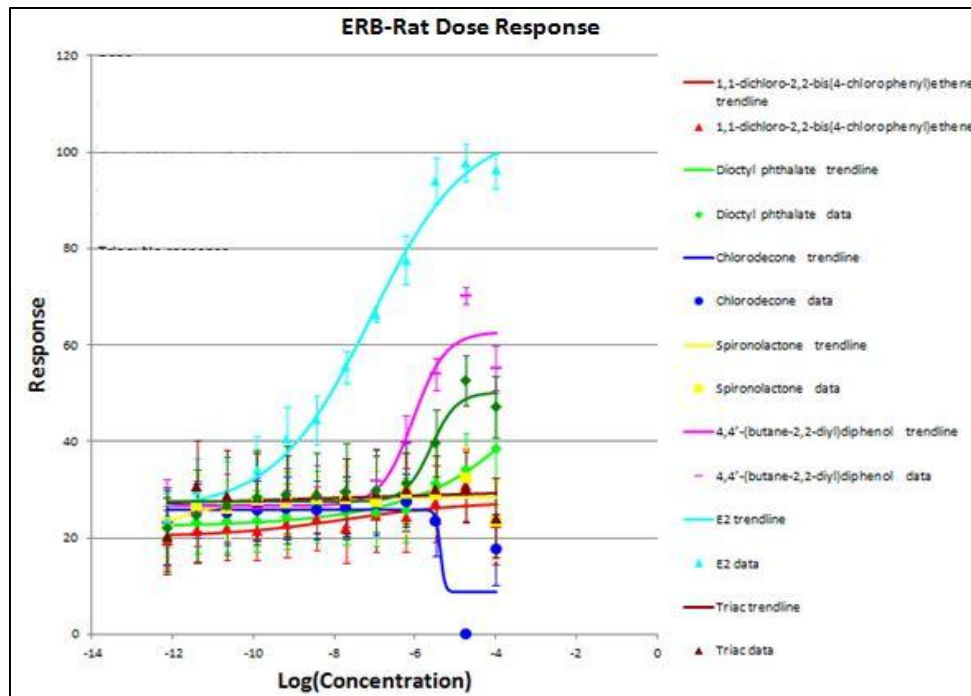


Figure 9: Dose response curve for ER β -Rat

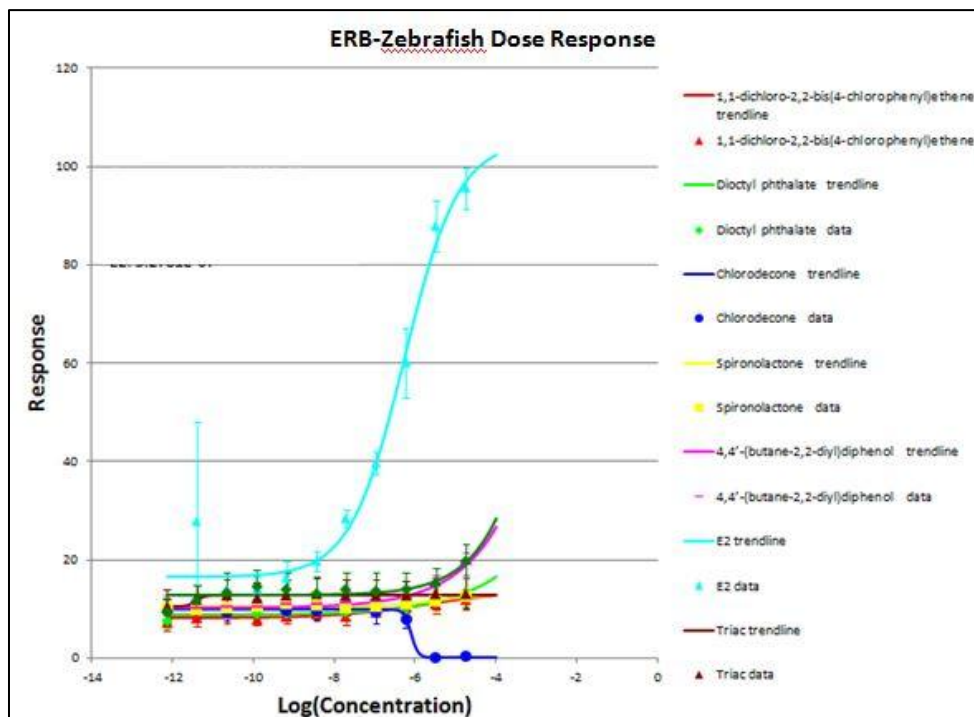


Figure 10: Dose response curve for ER β -Zebrafish

As seen in Figures 7-10, all of the animals showed a strong agonistic response to E2 (aqua curve), which is expected as E2 is the native ligand for ER β , and an antagonistic

response to chlorodecone (dark blue). However, not all of the animals responded to the other ligands as evidenced by the difference in response between zebrafish (Figure 10) and the mammals (Figures 8-10) to Bisphenol B (magenta) and BPA (dark green). The zebrafish ER β biosensor does not identify the two compounds as having any estrogenic activity while the other three mammalian sensors clearly show obvious responses to both compounds with Bisphenol B resulting in responses of greater than 40% and BPA resulting in responses greater than 30%. Thus, it is likely that weaker estrogenic compounds in particular induce species-specific response and that the response may be slightly dependent on whether the animal is cold or warm-blooded. A summary of the estrogenicity of the 15 ICCVAM compounds tested against the animal biosensors can be found in Table 2 on the next page.

Table 2: Summary of ICCVAM compound estrogenicity

Compound	ERβ-human	ERβ-cow	ERβ-rat	ERβ-zebrafish
1,1-Dichloro-2,2-bis(4-chlorophenyl)ethene	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Diocetyl phthalate	Weak ER agonist	Not estrogenic	Weak ER agonist	Not estrogenic
Chlorodecone	ER antagonist	ER antagonist	ER antagonist	ER antagonist
Spironolactone	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Bisphenol B	ER agonist	ER agonist	ER agonist	Weak ER agonist
Cycloheximide	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Progesterone	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Linuron	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
2,4,5-Trichlorophenoxyacetic acid	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
3,4-Cumyl phenol	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
6-Propyl-2-thiouracil	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Atrazine	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Cyproterone acetate	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic
Flavone	Not estrogenic	Not estrogenic	Not estrogenic	Not estrogenic

Out of the 15 compounds, it was found that chlorodecone (kepone) demonstrated antagonistic activity in all animals, which is consistent with studies using rats showing that chlorodecone inhibits the binding of estradiol to the estrogen receptor in a competitive manner [21]. Two other compounds, Bisphenol B and dioctyl phthalate also showed weak agonistic behavior using the mammalian sensors, which is supported with literature results of phthalate esters being known EEDCs and the fact that Bisphenol B is

structurally similar to BPA, another weak agonist [2]. The fact that Bisphenol B and chlorodecone induced responses in all of the mammalian biosensors suggests that the ER β biosensors are correctly identifying estrogens as both Bisphenol B and chlorodecone were shown to bind the ER receptor in *in vitro* binding assays [22].

Development of new sentinel species-based biosensors

Based on initial results using ER β biosensors for cow, rat, zebrafish, and human, it was desired to expand the biosensor library to include sentinel species such as reptiles, amphibians, and birds in order to further investigate the dependence of ligand response on species. Sentinel species were also of interest due to their ability to serve as early indicators of environmental toxins, making them ideal candidates for early detection methods.

The sentinel species-based ER β biosensors were constructed through replacement of the human ER β LBD with the corresponding animal ER β LBD to produce pMIT::ER β *(zebra finch) and pMIT::ER β *(*L. sylvaticus*) constructs. An alligator ER β biosensor was also constructed, but it was cloned directly into the higher sensitivity construct, pMIT::ERB1GS*(alligator). Also, due to the presence of single nucleotide polymorphisms in the cDNAs for *L. sylvaticus* (LS), a total of four *L. sylvaticus* ER β biosensors were created and are denoted as LS ER β #2, LS ER β #4, LS ER β #5, and LS ER β #6. However, these results will focus only on LS ER β #2 and its higher sensitivity analog as it was found that the SNPs resulted in only silent mutations and had no significant effect on ligand response.

Following the high throughput bacterial biosensor assay method previously described, the sentinel species biosensors were validated against E2 as seen in Figure 11 on the next page.

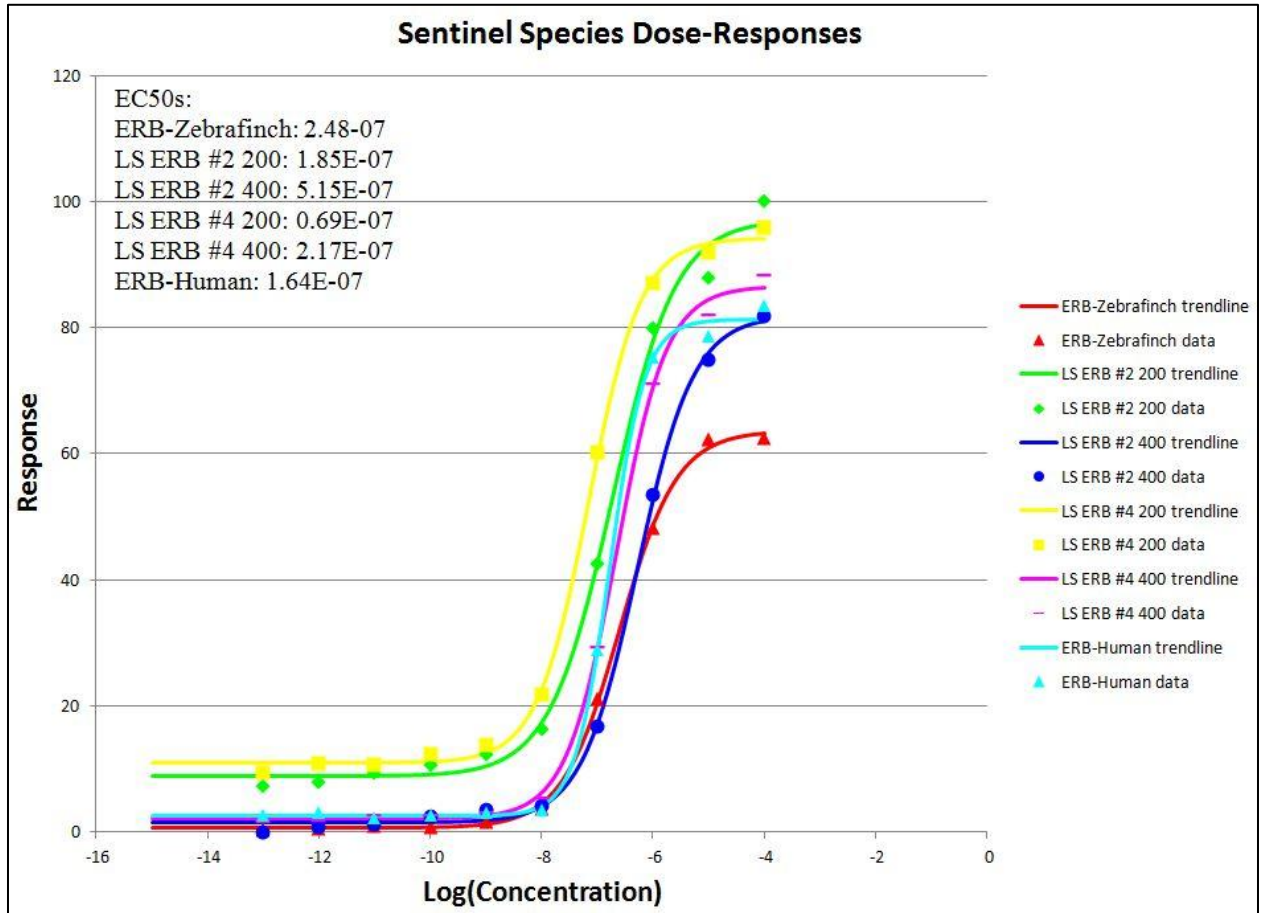


Figure 11: Dose response curves for ERβ-zebrafinch, LS ERβ #2, and ERβ-human

Both ERβ-zebrafinch and LS ERβ #2 were able to respond to E2 as seen in Figure 11 above, which suggests that the ERβ LBD was successfully cloned. Furthermore, the EC₅₀ values for E2 with all three animals were found to be very similar (ERβ zebrafinch: 2.48E-7, ERβ human: 1.64E-7, and ERβ #2 200: 1.85E-7), as expected since E2 is the native ligand for ERβ and thus a strong ER agonist. It was also found that a dilution of 1:200 for ERβ #2 was ideal for future biosensing tests in order to be consistent with the basal growth rates of the other animal biosensors.

Creation of higher sensitivity biosensors

Throughout the course of testing, it was found that several of the biosensors were unable to respond (reach saturation) to some of the ligands. Thus, higher sensitivity analogs of four animal-based biosensors were created to examine whether the increased sensitivity would enable the ligand response to reach saturation. The higher sensitivity versions of ER β -cow, ER β -zebrafish, ER β -alligator, and LS ER β #2 were constructed by adding a 1GS linker (5 extra amino acids consisting of 4 glycines and 1 serine) into the biosensor as shown in Figure 12 below.



Figure 12: Schematic at the DNA level of the 1GS linker biosensor

In the 1GS biosensor, the 1GS linker is inserted between the C-terminus of the intein and the TS reporter enzyme. The extra five amino acids help to relieve some of the steric hinderance experienced by the TS reporter enzyme by distancing it from the rest of the biosensor scaffold seen in Figure 3. This allows for an increased basal level of TS activity as TS dimerization is less hindered in this configuration. Thus, the sensitivity of the biosensor is enhanced as the effect of a confirmation change in the LBD on TS activity is amplified.

Similar to the original cloning of the animal-based ER β biosensors, the 1GS biosensors were constructed by swapping out the human ER β LBD in the pMIT::ER β 1GS*(human) construct with the desired animal ER β LBD. The higher

sensitivity analogs were then tested against several estrogens and compared to their non 1GS counterparts as seen in Figures 13 and 14 on the next page.

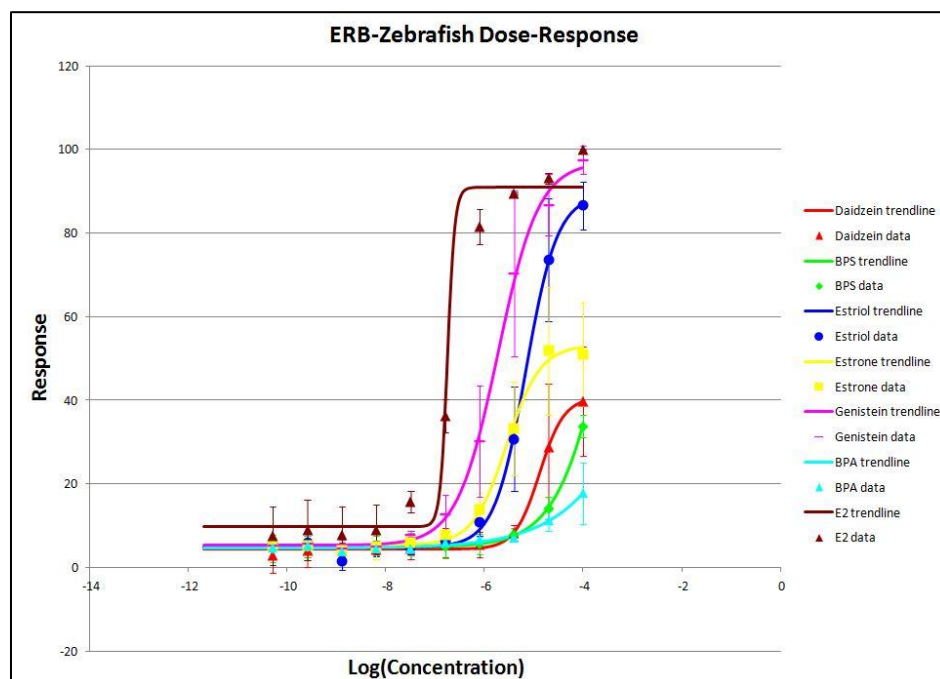


Figure 13: Dose response curve for ERβ-zebrafish against seven estrogens

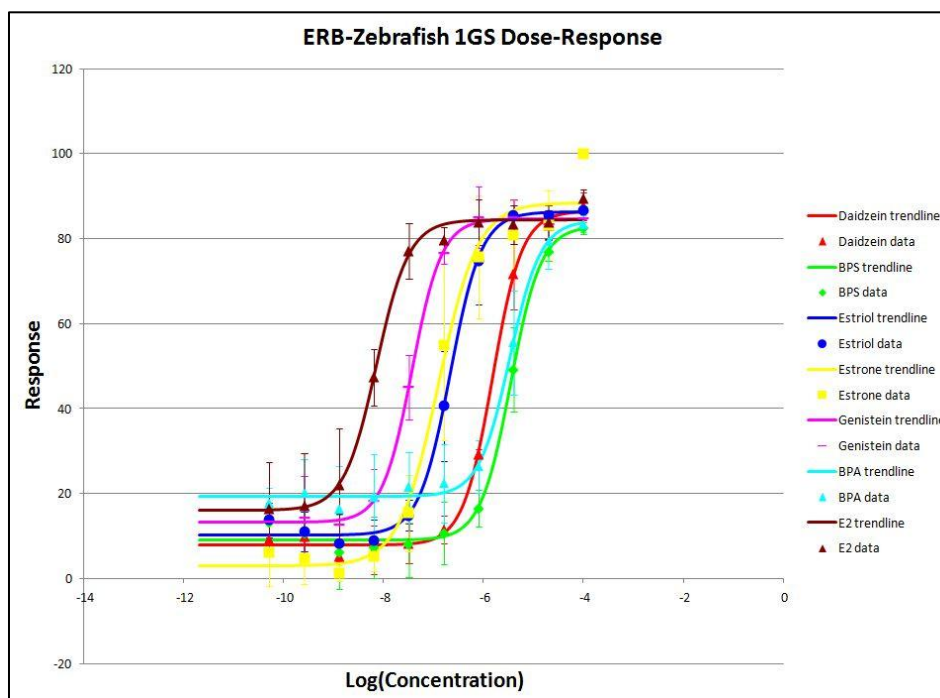


Figure 14: Dose response curve for ERβ-zebrafish 1GS against seven estrogens

As seen in Figure 13, ER β -zebrafish was unable to respond to BPA and BPS, which are both weak ER agonists, as evidenced by the lack of saturation in the dose response curves. Additionally, the response curves for Daidzein, Genistein, and Estriol appear to be just barely reaching saturation even at the highest concentration of 1E-4 M. The response levels of the three weakest estrogens, Daidzein, BPS, and BPA are also all under 40%, suggesting that the ER β -zebrafish biosensor is not sensitive enough to confidently assess these estrogens. However, in comparing the results of the ER β -zebrafish 1GS biosensor tested with the same ligands, there is an obvious and distinct improvement in sensitivity with the addition of the 1GS linker. The ER β -zebrafish 1GS biosensor dose response curves in Figure 14 indicate that all of the ligands were able to reach saturation and even more surprisingly, the response levels of BPA and BPS were increased to above 80% from below 20% and below 40% respectively. This enhancement in sensitivity was observed across all animal species and ligands, although the effect was not as pronounced for stronger estrogens and mammalian species. Table 3 below shows the EC₅₀ values of seven estrogens using the ER β and ER β 1GS biosensors of each animal.

Table 3: EC₅₀ values for ER β and ER β 1GS biosensors

Compound	Human (μ M)	Human 1GS (μ M)	Cow (μ M)	Cow 1GS (μ M)	Zebrafish (μ M)	Zebrafish 1GS (μ M)	LS #2 (μ M)	LS #2 1GS (μ M)
E2	0.35	0.06	0.34	0.16	0.41	0.01	0.62	0.02
Genistein	0.05	0.02	0.14	0.06	3.86	0.05	0.15	0.01
Daidzein	0.59	0.33	4.63	0.21	10.90	1.84	1.57	0.07
Estriol	1.02	0.36	1.29	0.60	7.41	0.25	1.06	0.30
Estrone	1.26	0.30	0.51	0.13	3.26	0.15	1.32	0.20
BPA	6.70	1.33	8.31	0.73	N/A	4.81	12.27	2.00
BPS	2.07	1.50	3.51	1.03	N/A	5.77	33.99	1.16
4,4	1.22		1.62	0.20	N/A	1.67	4.97	0.69

Addition of the 1GS linker in the zebrafish biosensor resulted in more than 20-fold increases in sensitivity for every compound except for Daidzein, which experienced

only a 5-fold increase. However, with the mammalian-based sensors, the average increase in sensitivity was only approximately 2 to 5-fold. As seen in Table 3, the greatest increases in sensitivity were observed with the weaker estrogens, regardless of species. This was expected though since the 1GS linker was included specifically to amplify the effect of weaker estrogens.

Comparison of ligand response across species

Using the sentinel species-based ER β biosensors and existing animal biosensors, a comprehensive screen of nine different estrogens of varying strength was conducted in order to understand how ligand response varies across species. All of the ER β biosensors were tested using the high throughput method and RPTA and EC₅₀ values were calculated from the resulting dose response curves. A comparison of EC₅₀ values normalized to the human response can be seen in Figure 15 on the next page.

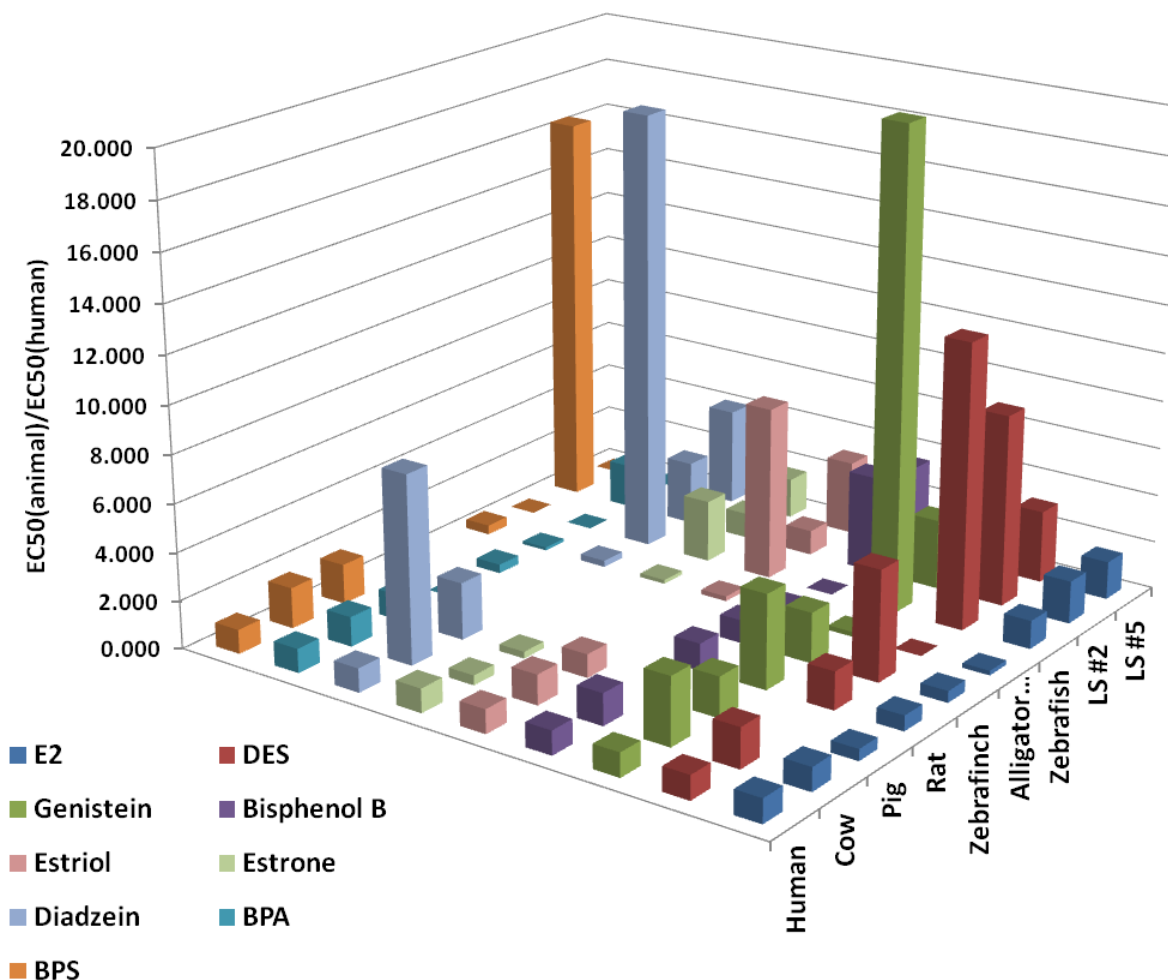


Figure 15: Comparison of relative EC₅₀ values normalized to the human response

Figure 15 depicts the relative EC₅₀ values of each compound across each species after normalization to the human response such that all of the human responses are equal to 1. As seen in Figure 15, the greatest deviations in ligand response occur with the cold-blooded animals. This difference could be a result of the genetic and metabolic differences between the two sets of animals due to the unique environments that each live in. Studies have shown that zebrafish have a lower sensitivity to BPA as compared to rainbow trout due to the ability of the zebrafish liver to quickly metabolize BPA. Differences in estrogen sensitivities have also been attributed to differences in estrogen receptor (ER) amino acid structure across species and differential subtype ER tissue

distribution [23]. The EC₅₀ values also show that warm-blooded animals tend to respond similarly to each other while cold-blooded animals tend to respond similarly to other cold-blooded animals. Thus, animals that live in the same environment (i.e. on land versus in water) may have evolved to have similar responses due to similarities in their ER amino acid structures since they likely share common ancestors.

The EC₅₀ values for cold-blooded animals are also observed to be higher than those for warm-blooded animals, suggesting that cold-blooded creatures are less sensitive to estrogens in general as compared to warm-blooded creatures. Out of the nine species, zebrafish displayed the greatest deviations from human response with its responses to four compounds (DES, Genistein, Daidzein, and Estriol) having the highest EC₅₀ values, suggesting that it is the least sensitive to these compounds. It is also interesting to note that out of all the species, alligator displayed the greatest sensitivity to the different estrogens with the lowest EC₅₀ values for a majority of the estrogens. This can be attributed though to the fact that the alligator is a species that experiences temperature-dependent sex determination and is thus, particularly susceptible to environmental perturbations [23].

Aside from cross-species comparisons, testing of the ER β biosensors against the nine estrogens also enabled direct comparison of the EC₅₀ values determined using the bacterial biosensor against ICCVAM's *in vitro* ER binding and transcriptional activation assays as seen in Table 4 on the next page.

Table 4: Comparison of EC₅₀ values using the ER β biosensor and the ICCVAM *in vitro* binding assay

Compound	Human ER β bacterial biosensor EC ₅₀ (μ M)	ICCVAM <i>in vitro</i> assay EC ₅₀ (μ M)
DES	0.0137	0.0189
E2	0.349	0.098
Estrone	1.26	0.630
Estriol	1.06	34.8
BPA	6.70	450

As seen in Table 4, the EC₅₀ values found using the ER β biosensor for E2, DES, and Estrone are very similar to the values determined using the ICCVAM *in vitro* assay with only a 2-fold difference for Estrone and a 3-fold difference for E2. However, there is a significant difference in EC₅₀ values between the two methods for Estriol and BPA, the weakest estrogens of the five. The ER β biosensor assay method resulted in lower EC₅₀ values (1.06 and 6.70 μ M) for the two compounds as compared to the ICCVAM assay (34.8 and 450 μ M), suggesting that the ER β biosensor is more sensitive for weaker estrogens.

In addition to EC₅₀ values, RPTA values, which can be viewed as a measure of the binding affinity of the ligand relative to E2, were also determined. The RPTA values for all of the estrogens except Genistein and DES can be found in Figure 16 on the next page. Genistein and DES were excluded from this figure as both had RPTA values significantly greater than 1 as both were found to have greater binding affinity for ER β than E2.

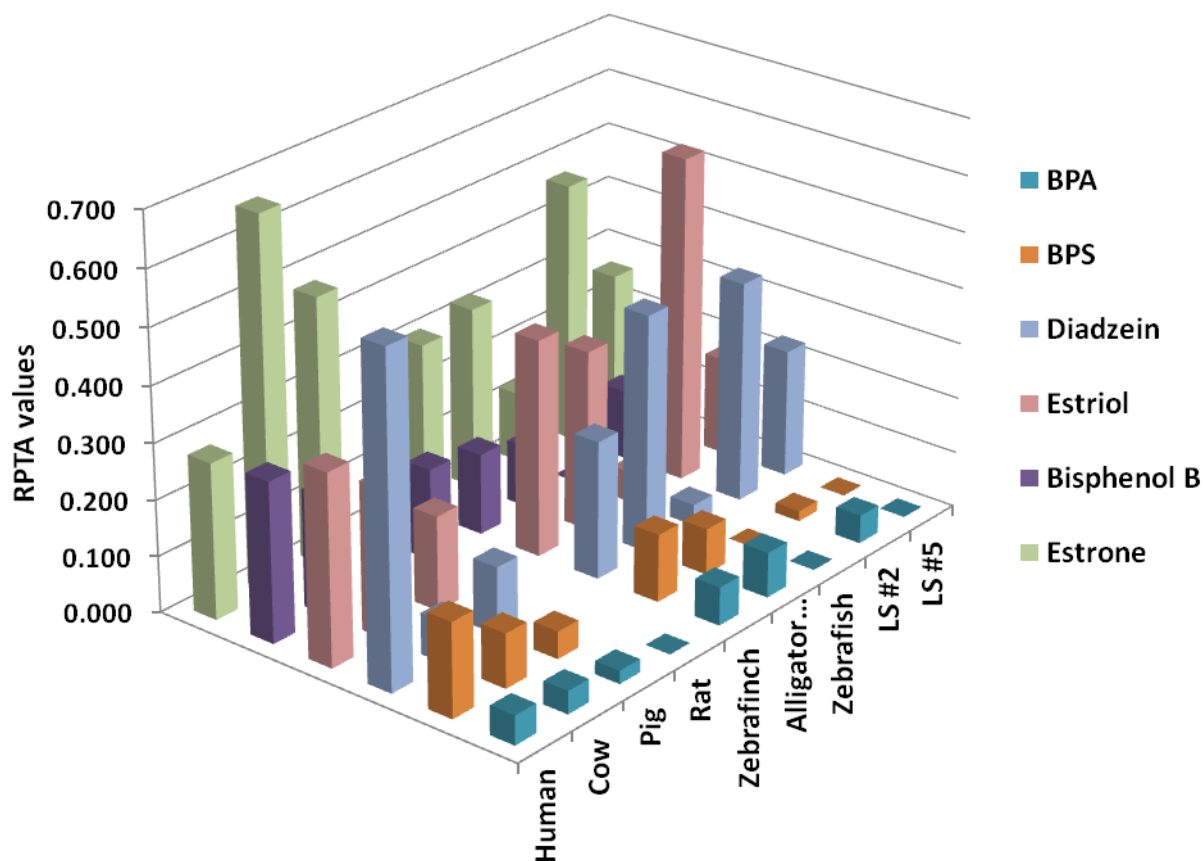


Figure 16: RPTA values of the remaining six estrogens (BPA, BPS, Estrone, Estriol, Diadzein, and Bisphenol B)

As seen in Figure 16, the RPTA values for all of the shown estrogens are lower than 1. Thus, all of these compounds bind to the ER β receptor less strongly as compared to E2, making them weaker ER agonists. BPS and BPA were found to have the lowest RPTA values, suggesting that these compounds are the weakest ER agonists of the six. This effect was also observed across all species, suggesting that the decreased sensitivity to these compounds was not a result of differences in the ER amino acid structure across various species. Figure 16 also demonstrates how species response greatly deviates with weaker estrogens as there is no clear trend observable nor is it possible to determine a general ranking of estrogenicity for Daidzein, Estriol, Estrone, and Bisphenol B. Table 5,

shown below, displays a ranking of compounds based on RPTA values for each species and further emphasizes this point.

Table 5: Ranking of estrogens by RPTA value for each biosensor

Species	Highest RPTA	2nd Highest RPTA	3rd Highest RPTA	4th Highest RPTA	5th Highest RPTA	6th Highest RPTA	7th Highest RPTA	8th Highest RPTA	Lowest RPTA
Human	DES	Genistein	E2	Diadzein	Estriol	4,4	Estrone	BPS	BPA
Human 1GS	Genistein	E2	Estrone	Diadzein	Estriol	BPA	BPS		
Cow	DES	Genestein	E2	Estrone	Estriol	4,4	BPS	Diadzein	BPA
Cow 1GS	Genestein	Estrone	E2	4,4	Diadzein	Estriol	BPA	BPS	
Zebrafish	DES	E2	Estrone	Genestein	Estriol	Diadzein	4,4 = BPS = BPA = No response		
Zebrafish 1GS	DES	E2	Genestein	Estrone	Estriol	4,4	Diadzein	BPS=BPA	
Rat	DES	Genestein	E2	4,4	BPA				
Pig	Genestein	E2	Estrone	Estriol	Diadzein	BPS	BPA		
Zebrafinch	DES	Genestein	E2	Estriol	Estrone	Diadzein	4,4	BPS	BPA
Alligator 1GS	DES = no response	Genestein	E2	Diadzein	Estrone	Estriol	4,4	BPS	BPA
LS #2	DES	Genestein	E2	Estriol	Estrone	Diadzein	4,4	BPA	BPS
LS #2 1GS	Genestein	E2	Diadzein	Estrone	Estriol	4,4	BPS	BPA	
LS #5	DES	Genestein	E2	Estrone	Daidzein	Estriol	4,4	BPS = BPA = No response	

Examination of the RPTA values reveals that strong ER agonists such as DES and Genistein induce fairly consistent responses across all species with DES being observed to have the highest RPTA values followed by Genistein and E2. The remaining six estrogens result in RPTA rankings that greatly vary depending on species, making it difficult to rank the compounds in order of estrogen strength. However, it is likely that Estrone, Estriol, and Diadzein are the next strongest ER agonists after E2 and that BPA is the weakest ER agonist based on their consistency in RPTA rankings across all of the various species.

Adaption to other nuclear hormone receptors

In addition to the creation of novel sentinel species-based ER β biosensors, biosensors selective for the thyroid receptor (TR β) and estrogen receptor alpha (ER α) were also studied. Due to the highly conserved nature of nuclear hormone receptors across species and the modular design of the bacterial biosensor, *L. sylvaticus* and *L.*

pipens TR β bacterial biosensors were also created. The TR β biosensors were constructed similar to how the *L. sylvaticus* ER β biosensors were by performing protein sequence alignment of the *L. sylvaticus* and *L. pipens* TR β LBDs with the human TR β LBD. The human TR β LBD in pMIT::TR β *(human) was then replaced with the *L. sylvaticus* and *L. pipens* TR β LBDs via overlap extension PCR to yield pMIT::TR β *(*L. sylvaticus*) and pMIT::TR β *(*L. pipens*). The new TR β biosensors were then tested with tiratricol (Triac), which is a thyroid hormone analog and dose-response curves were produced as shown in Figure 17 below.

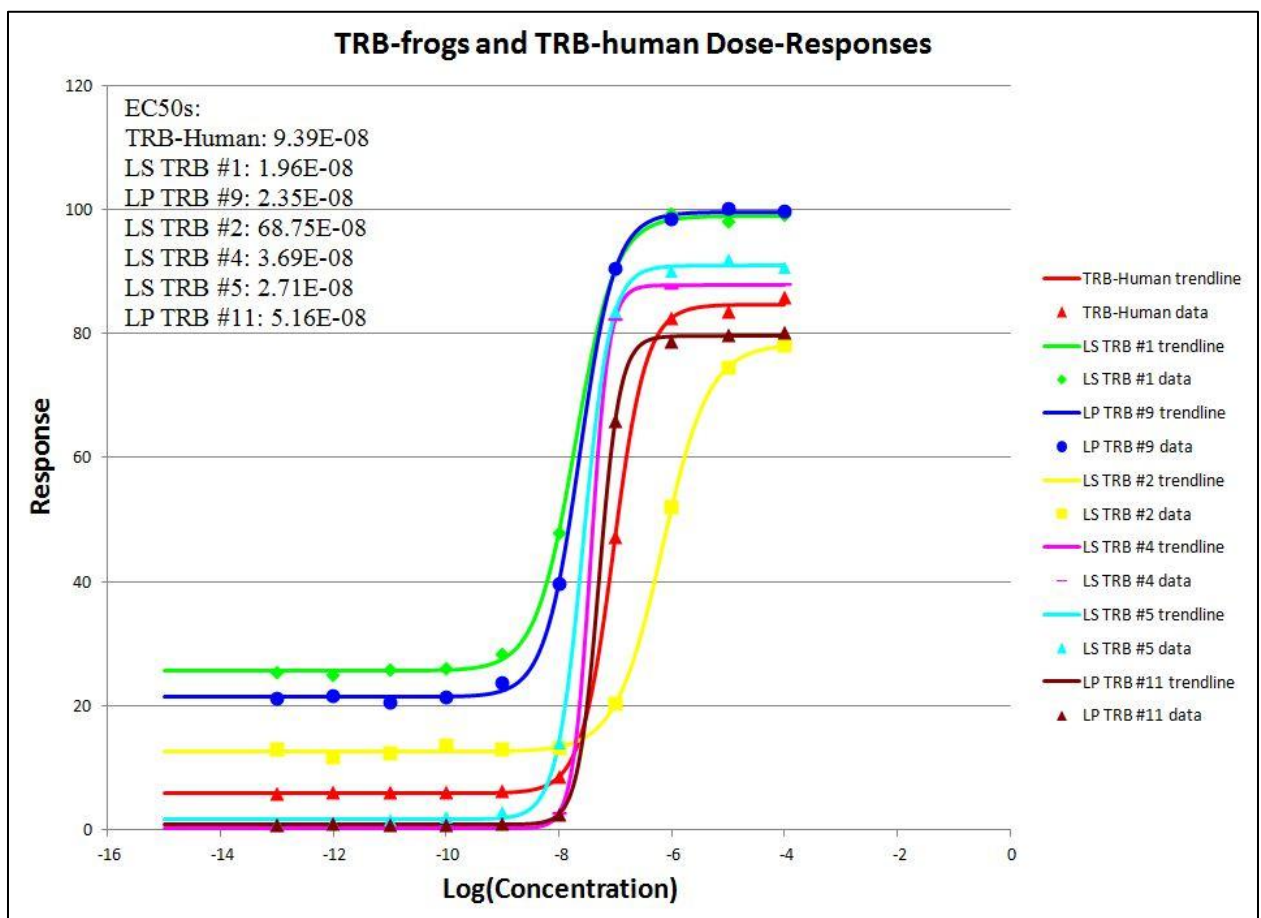


Figure 17: Dose-response curves of *L. sylvaticus* (LS), *L. pipens* (LP), and human TR β biosensors

As seen in Figure 17, all of the TR β biosensors were able to respond to Triac, which is expected since Triac is an agonist for the thyroid receptor. There are six different LS and LP TR β biosensors shown due to the existence of SNPs in the cDNAs. All of the frog TR β EC₅₀ values, with the exception of LS TRB #2 were lower than that of human TR β , indicating that the frogs are slightly more sensitive to Triac as compared to human. The fact that LS TRB #2 exhibited such a high EC₅₀ value of 68.75E-8 M compared to an average of 2.78E-8 M for the other LS sensors and 9.39E-8 M for human could be attributed to the existence of a SNP that may have caused an amino acid change in the LBD. Overall though, the majority of the frog biosensors responded approximately the same to Triac regardless of the species (LS or LP) or the presence of SNPs.

It was also desired to create ER α analogs of the animal biosensors to investigate the difference in ligand response across the two receptor sub-types as it is known that ER α and ER β are primarily located in different tissues, and thus have different roles in development [1]. Thus, cloning of ER α analogs of the ER β biosensors for *L. pipens* was also performed and pMT::ER α *(*L. pipens*) sensors were constructed. Although the *L. pipens* ER α biosensors have been verified via digest check and PMGF sequencing, they have yet to be validated against a library of known estrogens.

Conclusion

Testing of the cow, rat, zebrafish, and human ER β bacterial biosensors against a library of 15 compounds comprised of estrogen agonists, antagonists, and non-estrogens revealed that the ER β bacterial biosensor high-throughput method is capable of reliably detecting estrogenic compounds within 16 -22 hours after ligand introduction. In addition, species-specific ligand responses were observed, especially for the weaker

estrogens and for cold-blooded animals compared to warm-blooded animals. Out of 15 ICCVAM recommended compounds, it was found that chlorodecone demonstrated ER antagonistic activity, and that two compounds, Bisphenol B and dioctyl phthalate, showed weak ER agonistic behavior using the mammalian-based biosensors, which was consistent with the results of other studies and ICCVAM's *in vitro* ER binding assay results. The agonistic effects of the aforementioned two ligands though were found to be too weak to be observed using the zebrafish ER β biosensor. Due to this lack of sensitivity in the earlier tests, it will be necessary to re-examine these ligands using the 1GS biosensors to verify their estrogenicity and to determine if any additional estrogenic compounds were missed during the initial ICCVAM compound screening.

As a result of the species-dependent responses observed during ICCVAM testing, three novel sentinel species-based ER β bacterial biosensors, pMIT::ER β 1GS*(alligator), pMIT::ER β *(zebra finch), and pMIT::ER β *(LS) were constructed. These ER β biosensors were validated using known strong and weak estrogen agonists such as DES, Daidzein, E2, Genistein, and BPA and it was found that all three biosensors could reliably detect most, if not all of the estrogens. In addition, higher sensitivity analogs (denoted as pMIT::ER β 1GS*(animal) or ER β -animal 1GS) were created of the cow, zebrafish, LS #2, and alligator ER β biosensors through the insertion of an additional five amino acids (1GS linker) between the C-terminus of the intein and the TS reporter enzyme in the biosensor scaffold. The addition of the 1GS linker was found to improve ligand sensitivity by anywhere from 1 to about 77-fold depending on the species and ligand. The 1GS biosensors also enabled determination of EC₅₀ values for compounds such as BPA and BPS by improving sensitivity and enabling saturation of the ligand response. Prior to

construction of the 1GS biosensors, EC₅₀ values for these compounds could not be determined for some animals. In addition to the creation of sentinel species-based ER β biosensors and their higher sensitivity analogs, TR β and ER α biosensors were also constructed for *L. sylvaticus* and *L. pipens*. Validation and future testing of the TR β and ER α biosensors against their respective compound libraries though will need to be performed to truly assess the ability of the ER β biosensor method to be adapted to other nuclear hormone receptors.

Through analysis of the EC₅₀ and RPTA values generated from the nine different animal-based biosensors, it was found that ligand responses are dependent on the species and on the ligand itself. In general, it was observed that cold-blooded animals (amphibians and fish) were less sensitive to the estrogens as compared to mammals and that like-blooded animals tended to behave similarly. The only exception to these observations was discovered to be ER β -alligator, which can be attributed to the fact that alligators are more susceptible to environmental changes than other animals. Analysis of the RPTA values was also consistent with the earlier observation that weaker estrogens are more prone to deviations in ligand response across various animal species.

Overall, development and testing of novel animal-based ER β biosensors revealed the strength of the bacterial biosensor method as a fast, inexpensive, and alternative process for determining estrogenic compounds. The ER β bacterial biosensors were able to correctly identify Genistein, DES, E2, Daidzein, Estrone, Estriol, and BPA as estrogen agonists and provide estimates of relative estrogenicity that were comparable to *in vitro* binding and transcriptional activation assays. Differences in EC₅₀ values between the two assays were observed to be less than half an order of magnitude for strong ER agonists

(E2, DES, and Estrone), but increased to up to two orders of magnitude for weaker ER agonists (BPA and Estriol). In addition, the ER β bacterial biosensor was able to determine ER agonist or antagonist behavior within only 16-22 hours after ligand introduction as compared to days or weeks with animals. Furthermore, the ER β biosensor method is easily adaptable to other nuclear hormone receptors and animals through its modular construction, enabling the potential to create biosensors selective for any animal and NHR desired. Expansion of the ER β biosensor library to include sentinel species and higher sensitivity constructs also provided a broader understanding of how ligand response deviates across species and allows for the ability to better prevent human and wildlife health risks through early detection of environmental endocrine disruptors.

Future Work

Through development and screening of the animal-based bacterial biosensors, it was possible to demonstrate the potential of the bacterial biosensor to be used as an alternative technology for preliminary drug screening and as a tool to gain a greater understanding of species-specific susceptibility to endocrine disruptors. Despite these promising results though, much work remains before the bacterial biosensor could truly be implemented as a common screening method.

As mentioned earlier, some of the preliminary studies using the ER β biosensors were inconclusive due to a lack of sensitivity. Thus, the remainder of the animal-biosensors (zebrafinch, rat, sole, and pig) will be moved to the 1GS construct in order to create higher-sensitivity analogs of every animal-based ER β biosensor. Once the 1GS biosensors have been constructed and validated, a comprehensive screen of the nine estrogens will be performed again to obtain a clearer understanding of how ligand

response varies with the species and the strength of the ligand. Screening of the ICCVAM recommended compounds will also be repeated with the 1GS biosensors to identify additional ER agonists and antagonists as prior studies were unable to detect some of the weaker ER agonists.

In addition to conducting further tests with the 1GS biosensors using larger and more diverse compound libraries, adaption of the ER β biosensor to other pharmaceutically relevant nuclear hormone receptors will also be continued in order to demonstrate the biosensor's versatility. TR β and ER α biosensors have already been designed, but these sensors still must be validated and tested. Construction of a glucocorticoid biosensor will also be an excellent addition to the biosensor library as the glucocorticoid receptor is involved in numerous metabolic and immune response-related diseases. Finally, an *L. pipens* ER β biosensor will be constructed as well as it would be interesting to investigate the differences in ligand response across the two frog species since all of our previous work has focused on differences between distinct genera.

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